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1 **TITLE:**
2 **Isolation, Culture, Characterization, and Differentiation of Human Muscle Progenitor Cells from**
3 **the Skeletal Muscle Biopsy Procedure**
4
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27
28 **SUMMARY:**
29 We present techniques for isolating, culturing, characterizing, and differentiating human primary
30 muscle progenitor cells (hMPCs) obtained from skeletal muscle biopsy tissue. hMPCs obtained
31 and characterized through these methods can be used to subsequently address research
32 questions related to human myogenesis and skeletal muscle regeneration.
33

34 **ABSTRACT:**
35 The use of primary human tissue and cells is ideal for the investigation of biological and
36 physiological processes such as the skeletal muscle regenerative process. There are recognized
37 challenges to working with human primary adult stem cells, particularly human muscle
38 progenitor cells (hMPCs) derived from skeletal muscle biopsies, including low cell yield from
39 collected tissue and a large degree of donor heterogeneity of growth and death parameters
40 among cultures. While incorporating heterogeneity into experimental design requires a larger
41 sample size to detect significant effects, it also allows us to identify mechanisms that underlie
42 variability in hMPC expansion capacity, and thus allows us to better understand heterogeneity in
43 skeletal muscle regeneration. Novel mechanisms that distinguish the expansion capacity of

44 cultures have the potential to lead to the development of therapies to improve skeletal muscle
45 regeneration.
46
47 **INTRODUCTION:**
48 Skeletal muscle is the largest organ system in the human body, accounting for 30–40% of whole
49 body mass[1]. In addition to its well-recognized role in locomotion, skeletal muscle maintains body
50 temperature and posture, and plays a central role in whole body nutrient homeostasis. Research
51 involving human participants, animals, and cell culture models are all valuable to address
52 questions pertaining to skeletal muscle biology and regeneration. Isolation and culture of human
53 primary muscle progenitor cells (hMPCs) provides a robust model that allows for cell culture
54 techniques and manipulations to be applied to human samples. An advantage of using hMPCs is
55 that they retain the genetic and metabolic phenotype from each donor[2,3]. Maintenance of the
56 donor phenotype allows researchers to examine inter-individual variation in the myogenic
57 process. For example, we have employed our hMPC characterization method to identify age- and
58 sex-related differences in hMPC population expansion capacity[4].
59
60 The purpose of this protocol is to detail techniques to isolate, culture, characterize, and
61 differentiate human primary muscle progenitor cells (hMPCs) from skeletal muscle biopsy tissue.
62 Building on previous work that described hMPCs and identified potential cell surface makers for
63 hMPC isolation[5,6], this protocol fills a critical gap in knowledge by linking the isolation to the
64 characterization of hMPCs. Further, the detailed step-by-step instructions included in this
65 protocol make hMPC isolation and characterization accessible to a broad scientific audience,
66 including those with limited prior experience with hMPCs. Our protocol is among the first to
67 describe use of an imaging cytometer to track cell populations. Newly designed imaging
68 cytometers are state-of-the-art, high-throughput, and microplate-based, enabling live cell
69 imaging, cell counting, and multichannel fluorescence analysis of all cells in each well of a culture
70 vessel within minutes. This system allows for rapid quantification of dynamic changes in
71 proliferation and viability of an entire cell population with only minimal disruption to the culture.
72 For example, we are able to perform objective measures of confluence on successive days in vitro
73 to determine growth kinetics of each culture derived from different donors. Many protocols in
74 the literature, particularly those involving the differentiation of MPCs, require cells to reach a
75 defined level of confluence before initiating differentiation or treatment[7]. Our method allows for
76 objective determination of the confluence of each well in a culture vessel allowing researchers
77 to initiate treatment in an unbiased, non-subjective manner.
78
79 In the past, a major limitation of using primary hMPCs was low yields that limit the number of
80 cells available for experiments. We and others have shown the yield of MPCs from skeletal muscle
81 biopsy tissue is 1–15 MPCs per milligram of tissue (**Figure 1**)[8]. Because our protocol allows for
82 four passages of the cells prior to purification with fluorescence activated cell sorting (FACS), our
83 cryopreserved hMPC yields, derived from small amounts of biopsy tissue (50–100 mg), are
84 sufficient to address research aims where multiple experiments are required. Our FACS protocol
85 produces a ~80% pure (Pax7 positive) MPC population, thus our protocol is optimized for both
86 yield and purity.
87

88 **PROTOCOL:**
89
90 This protocol was approved by the Institutional Review Board at Cornell University. All
91 participants were screened for underlying health conditions and gave informed consent.
92
93 **1. Obtaining human muscle tissue via the skeletal muscle biopsy**
94
95 1.1. Identify the vastus lateralis muscle via palpation, anatomical landmarks, and active
96 contraction of the muscle.
97
98 1.2. Palpate the vastus lateralis by having the participant tighten their quadriceps muscle and
99 find the belly of the muscle, about 1/3 of the way from the patella to the hip, and just
100 ventrolateral to the femur.
101
102 1.3. Use a paper measuring tape to mark an area with a surgical marker about 4−6 inches from
103 the top of the patella representing the belly of the muscle.
104
105 1.4. Shave any hair near the marked area and then cleanse with a surgical grade antiseptic that
106 is used before medical procedures (For example: 2% chlorhexidine gluconate (CHG)/
107 70% isopropyl alcohol (IPA)) .
108
109 1.5. Anesthetize the biopsy site with 1% lidocaine.
110
111 NOTE: Avoid injecting the lidocaine into the skeletal muscle.
112
113 1.6. Make a 4−5 mm incision pathway at the marked site.
114
115 1.7. After the area is anesthetized, push a Bergstrom needle with trocar through the fascia into
116 the belly of the muscle (approximately 2−3 cm).
117
118 1.8. Draw muscle tissue into the Bergstrom needle by withdrawing the cutting trocar 2−3 cm
119 while an assistant applies suction by withdrawing an attached 60 mL syringe plunger.
120
121 1.9. Close the cutting chamber.
122
123 1.10. Rotate the base of the biopsy needle a quarter turn and repeat the sequence of opening
124 the needle, applying suction, and closing the needle.
125
126 NOTE: Each time the needle is closed, a cut of muscle tissue should be obtained inside the
127 Bergstrom needle. During sample collection, the needle will remain in the thigh in the same
128 vertical position while it is rotated longitudinally through 360° to obtain each sample. This
129 sequence can be repeated 3−4 times.
130
131 1.11. Expel the collected biopsy tissue from the needle into a sterile nonadherent dressing pad.

132

1.12. Examine the tissue and remove any visible adipose and fibrotic tissues using sterile scalpel blades or tweezers.

1.13. Place a portion (50–100 mg) of tissue into a sterile tube containing CO_2-independent nutrient medium (e.g., Hibernate A).

NOTE: The weight of the tissue is determined as follows. First place the capped tube containing the nutrient medium on a scale and tare. Next place the tissue into the tube, recap the tube, and then weigh the tube containing the tissue in the nutrient medium.

1.14. Store the tube containing the tissue and nutrient medium at 4 °C until processing.

NOTE: Biopsy tissue should be processed within 48 hours of collection.

2. Isolating human muscle progenitor cells from biopsy tissue

2.1. Mince biopsy tissue.

2.1.1. Transfer the muscle tissue from the nutrient medium into a sterile Petri dish in a biological safety cabinet or similar sterile workplace.

2.1.2. Use sterile scalpel blades to mince tissue into ~1 mm^3 pieces.

2.2. Wash minced biopsy tissue to remove residual debris.

2.2.1. Transfer the muscle tissue into a 15 mL conical tube containing 10 mL of phosphate-buffered saline (PBS) and allow the tissue to settle via gravity.

2.2.2. Aspirate the supernatant being careful not to disturb the muscle tissue.

2.2.3. Add 10 mL of PBS to the washed tissue and allow the muscle tissue to resettle via gravity.

2.2.4. Aspirate the supernatant being careful not to disturb the muscle tissue.

2.2.5. Add 10 mL of low glucose Dulbecco's modified Eagle medium (DMEM) and allow the tissue to resettle via gravity.

2.2.6. Aspirate the supernatant being careful not to disturb the muscle tissue.

2.3. Incubate the tissue in digest media.

2.3.1. Resuspend the muscle tissue in 3 mL of digest media (2 mg/mL collagenase D in low glucose DMEM).

176

179

2.3.2. Incubate the tube containing the muscle tissue in a water bath at 37 °C for 30 min.

181

2.3.3. Triturate the muscle tissue suspension every 10 min using a 10 mL serological pipette.

183

2.3.4. Add 83 µL of additional digest media and 24 µL of dispase solution (0.76 mM calcium acetate, 1.39 mM sodium acetate, 13.91 mg/mL dispase II in low glucose DMEM). Return the suspension to the 37 °C water bath.

187

2.3.5. Triturate the muscle tissue suspension every 5–10 min with a wide bore pipet tip.

189

2.3.6. Continue trituration until a uniform slurry is achieved, and for no longer than 1.5 h to prevent over digestion.

192

NOTE: Sufficient mincing of the tissue and the enzymatic activity of collagenase and dispase can impact sample digestion. A uniform slurry about to pass through a normal pipette tip within minimal occlusion after 1.5 h. If a uniform slurry is not achieved after 1.5 h, double check that the tissue mincing is sufficient, and tissue is minced to the appropriate size (step 2.1.2). Additionally, the activity of the enzymes should be tested as lot to lot variation in enzymatic activity does occur.

199

2.4. Pellet cells and freeze in recovery media.

201

2.4.1. Add 6 mL of growth media (GM; 79% Ham's F12, 20% fetal bovine serum, 1% penicillin/streptomycin, 5 ng/mL basic fibroblast growth factor [bFGF], pH 7.4, passed through a 0.22 µm filter) to the muscle slurry.

205

2.4.2. Pipette the suspension through a 70 µm cell strainer into a 50 mL conical tube. Rinse the strainer with an additional 4 mL of GM.

208

NOTE: Sample can be transferred back into a 15 mL conical tube for ease of visualizing the pellet. A larger wash volume can be used if subsequent cell yields are low.

211

2.4.3. Centrifuge at 300 x g for 5 min at room temperature (RT).

213

2.4.4. Aspirate the supernatant being sure not to disrupt the pellet. Flick the tube to resuspend the pellet in the remaining media. If cells are not going to be cultured immediately proceed to step 2.4.5 for instructions on long-term storage, otherwise proceed to step 3.1.6.

217

NOTE: If immediate culture is desired, GM in cell culture plates should be pre-incubated (steps 3.1.1 and 3.1.2) before biopsy processing begins.

220

221   2.4.5. Add 1.5 mL of recovery cell culture freezing media (**Table of Materials**) to the recovered
222   pellet.

223

224   2.4.6. Transfer the slurry into a cryovial. Place the cryovial in an isopropanol-controlled rate
225   chiller overnight at -80 °C. Store cryovial at -80 °C until ready for culture.

226

227   NOTE: Cryovials containing slurry can be kept in liquid nitrogen for long term storage (greater
228   than 1 month). Using an isopropanol-controlled rate chiller allows cells to be reproducibly frozen
229   at a rate of 1 °C/ min resulting in optimal cell recovery rates as evidenced by viability after freeze
230   and increased likelihood of attachment to cell culture vessels[9]. Alternatively, a beaker containing
231   RT isopropanol, placed in a -80 °C freezer may be used.

232

233   **3. Culturing human muscle progenitor cells**

234

235   3.1. Thaw the muscle slurry.

236

237   3.1.1. Prior to thawing and seeding *h*MPCs, pre-equilibrate cell culture plates in GM. Add 250 µL
238   of GM to 4 wells of a collagen-coated (collagen I), 24-well cell culture plate. Fill the remaining
239   wells with 500 µL of sterile base media (i.e., F12) to prevent evaporation during long-term
240   culture.

241

242   NOTE: For biopsies between 50 g and 100 g, four wells of a 24-well plate are appropriate. If the
243   biopsy tissue is less than 50 g, two wells may be more appropriate. Residual debris and low cell
244   number prevents the use of a cell counting technique at this step; therefore, the exact seeding
245   density is not determined and varies slightly from biopsy to biopsy.

246

247   3.1.2. Incubate cell culture vessels with GM at 37 °C in 5% CO_2 for 1 h prior to seeding.

248

249   3.1.3. Remove cryovials from the -80 °C freezer and place in a 37 °C water bath; the liquid in the
250   tube should be completely submerged in water.

251

252   3.1.4. When thawing is almost complete (~5 min), transfer the cryovial to a sterile laminar flow
253   hood. Transfer the muscle slurry out of the cryovial tube and add to a 15 mL conical tube
254   containing 6 mL of pre-warmed GM at a rate of 1 mL/min.

255

256   3.1.5. Centrifuge the muscle slurry at 300 x *g* for 5 min at RT.

257

258   NOTE: A swinging bucket centrifuge can make it easier to visualize small pellets at this step but is
259   not essential.

260

261   3.1.6. Aspirate the supernatant being sure not to disrupt the pellet. Flick the tube to resuspend
262   the pellet in the remaining media and resuspend in 1 mL of GM.

263

264   3.1.7. Transfer 250 µL of the resuspended pellet (now considered an *h*MPC suspension) to each
265   of the 4 wells of the pre-incubated 24-well cell culture plate.

266
267   3.2. Culture and passage *h*MPCs.

268
269   3.2.1. Maintain *h*MPC cultures in GM at 37 °C in 5% CO_2.

270
271   NOTE: Stocks of GM without the bFGF (i.e., F12, antibiotics, FBS) are prepared in batches and
272   kept at 4 °C for up to two weeks. bFGF is added to GM on the day of use. Media containing bFGF
273   can be stored for 48 h at 4 °C.

274
275   3.2.2. Twenty-four hours after isolation, aspirate the GM, gently wash the culture vessel 2x with
276   pre-warmed GM, and add fresh GM.

277
278   NOTE: Twenty-four hours should provide *h*MPCs adequate time to attach; no *h*MPCs should be
279   removed after washing. This step will also remove remaining debris that was generated during
280   the cell harvest, making the vessel more amenable to accurate confluence scanning using an
281   imaging cytometer (section 5 below). After this initial media change, GM is changed every 48 h.

282
283   3.2.3. Passage *h*MPCs when 70% confluence is achieved or when they have remained on the
284   same culture dish for 10 days, whichever occurs first.

285
286   NOTE: 70% confluence is approximately 55,000 cells/cm^2.

287
288   3.2.4. To passage, add 250 µL of pre-warmed trypsin to each well of a 24-well cell culture plate
289   and incubate for ~5 min.

290
291   3.2.5. Tap the cell culture vessel on a firm surface to detach *h*MPCs. A light microscope can be
292   used to verify that the *h*MPCs have detached.

293
294   3.2.6. Transfer the trypsin/*h*MPC suspensions to 5 mL of GM in a 15 mL conical tube.

295
296   3.2.7. Centrifuge the *h*MPC suspension at 300 x *g* for 5 min at RT.

297
298   3.2.8. Remove *h*MPC suspensions from the centrifuge and place on ice in the sterile laminar flow
299   hood.

300
301   NOTE: Keeping *h*MPCs on ice during passing results in less cell aggregation.

302
303   3.2.9. Aspirate supernatant from *h*MPCs and gently resuspend pellet in 1 mL of GM.

304
305   3.2.10. Count cells using a hemocytometer or an automated cell counter.

306
307   NOTE: A 1:5 dilution of cell suspension to cell counting buffer is generally appropriate.

308
309   3.2.11. Seed *h*MPCs onto collagen-coated culture dishes containing pre-warmed GM at a density
310   of 3,500 cells per cm^2. A combination of 10 cm plates and 24-well plates is ideal. The 24-well
311   plates can be scanned daily on an imaging cytometer to monitor growth.
312
313   3.2.12. Follow the same procedure (starting at step 3.2.4.) for subsequent passages.
314
315   NOTE: Cell health and purity can be monitored across passages using a marker of cellular
316   senescence (e.g., β-galactosidase) and immunostaining for Pax7.
317
318   3.3. Cryopreserve the *h*MPCs.
319
320   3.3.1. Cryopreserve excess cells for later use to make the culture volume more manageable.
321
322   3.3.2. For cryopreservation, follow the trypsinization procedure described in steps 3.2.4.-3.2.9.
323
324   3.3.3. While the cell suspension is being centrifuged, prepare a mixture of 20% (e.g., 400 μL)
325   dimethyl sulfoxide (DMSO) and 80% (e.g., 800 μL) GM. Pipette up and down 20x to ensure
326   adequate mixing. Leave the mixture to rest on ice.
327
328   3.3.4. Based on cell count, dilute the *h*MPC suspension to 2 x 10^6/mL in GM.
329
330   3.3.5. Combine the *h*MPC suspension and the 20% sterile DMSO/80% GM mixture 50/50. The
331   final cryopreservation media is a combination of DMSO (10%) and GM (90%) containing 1 x 10^6
332   *h*MPCs/mL.
333
334   3.3.6. Place 1 mL of the *h*MPC suspension prepared in step 3.3.5 into as many cryovials as the
335   initial cell count allows. Then place aliquoted *h*MPCs in an isopropanol-controlled rate chiller in
336   a -80 °C freezer overnight.
337
338   NOTE: At this step, typically between 5 and 15 million cells are obtained, 30–60% of which are
339   identified as *h*MPCs by FACS.
340
341   **4. Isolating Pax7$^+$ human muscle progenitor cells via flow cytometry**
342
343   4.1. To prepare *h*MPCs from live cultures (step 3.2.11), trypsinize enough culture vessels for 1 x
344   10^6–2 x 10^6 total cells. Prepare cell suspensions as described in steps 3.2.4–3.2.9.
345
346   4.2. Prepare *h*MPCs from cryopreserved cultures (step 2.4.6 or 3.3.6).
347
348   4.2.1. Select 1–2 tubes containing ~1 x 10^6 passage four *h*MPCs for each donor.
349
350   NOTE: Using passage four *h*MPCs allows for adequate cell yield while still maintaining
351   proliferative potential until passage six (**Figure 2**).

352

353 4.2.2. Rapidly thaw cells by removing them from the -80 °C freezer and placing them in a 37 °C
354 water bath with the liquid in the tube completely submerged.

355

356 4.2.3. When thawing is almost complete (~5 min), transfer hMPCs to a sterile laminar flow hood
357 and transfer the contents of the cell suspension into 6 mL of pre-warmed GM in a 15 mL conical
358 tube at a rate of 1 mL/min.

359

360 4.2.4. Prepare cell suspensions as described in steps 3.2.7–3.2.9.

361

362 4.2.5. Resuspend each pellet in 3 mL of FACS buffer (500 mL PBS, 12.5 mL normal goat serum, 2
363 mL 0.5 M EDTA, pH 7.4, passed through a 0.22 μm filter).

364

365 4.2.6. Centrifuge the cell suspension at 300 x *g* for 5 min at RT.

366

367 4.2.7. Aspirate supernatant from hMPCs and gently resuspend pellet in 250 μL of FACS buffer;
368 place on ice.

369

370 4.2.8. Place 100 μL of resuspended cells in a 1.7 mL microcentrifuge tube and leave on ice to be
371 used as live/dead controls (step 4.5.1).

372

373 4.3. Stain hMPCs for cell surface markers.

374

375 4.3.1. Prepare the following antibody cocktail (amounts shown are for each culture, scale-up
376 appropriately): 5 μL CD56 (neural cell adhesion molecule [NCAM]; PE-Cy7-conjugated) and 5 μL
377 CD29 (β1-integrin; AlexFluor488-conjugated). The final antibody dilution is 1:50.

378

379 4.3.2. Add 10 μL of the antibody cocktail to each hMPC suspension and incubate on ice for 30
380 min in the dark.

381

382 4.3.3. Add 10 mL of FACS buffer to each suspension/antibody cocktail mixture.

383

384 4.3.4. Centrifuge each tube at 300 x g for 5 min at RT.

385

386 4.3.5. Resuspend the pellet in 300 μL of FACS buffer and transfer to a 5 mL round bottom
387 polystyrene tube.

388

389 4.3.6. Keep the suspension on ice and protected from light.

390

391 4.4. Prepare the compensation beads.

392

393 NOTE: Positive controls for each antibody and a negative control (unstained beads) should be
394 prepared using compensation beads. Compensation bead controls should be run on the flow
395 cytometer prior to the samples to be sorted. These controls allow for determination of how much

396 the fluorochrome from each antibody can be detected in the other's channel so that
397 compensation can be applied.

399 4.4.1. Vortex compensation beads.

401 4.4.2. In three 1.7 mL microcentrifuge tubes, add one drop (50 µL) of compensation beads.

403 4.4.3. Add 1 µL of each antibody to the appropriate tube, or none to the negative control.

405 4.4.4. Incubate in the dark for 15 min.

407 4.4.5. Add 1.5 mL of FACS buffer to each tube and vortex.

409 4.4.6. Centrifuge each tube at 300 x g in a benchtop centrifuge for 5 min at RT.

411 4.4.7. Aspirate supernatant and resuspend the pellet in 200 µL of FACS buffer.

413 NOTE: The pellet may be very difficult to visualize; care should be taken when aspirating the
414 supernatant.

416 4.4.8. Place the suspension in a 5 mL round bottom polystyrene tube and then keep the tube on
417 ice and protected from light.

419 4.5. Prepare live and dead controls.

421 4.5.1. Divide the 100 µL of hMPCs from step 4.2.8 into two 1.7 mL microcentrifuge tubes (one for
422 the live control and one for the dead control).

424 4.5.2. Place the dead control tube in a heating block pre-warmed to 70 °C for >10 min. During
425 this time, keep the live control on ice.

427 4.5.3. Centrifuge each tube at 300 x g in a benchtop centrifuge for 5 min at RT.

429 4.5.4. Aspirate supernatant and resuspend the pellet in 150 µL of FACS buffer.

431 4.5.5. Combine the live/dead controls into a single 1.7 mL microcentrifuge tube.

433 4.6. Perform viability staining.

435 4.6.1. Add 1 µL of 7-aminoactinomycin D (7-AAD) to all tubes containing cells to be sorted and
436 the live/dead control.

438 4.6.2. Add GM to collagen-coated 24-well (250 µL/well) and 10 cm (8 mL/well) plates and allow
439 to equilibrate in a cell culture incubator at 37 °C in 5% CO_2.

440

441 4.7. Sort *h*MPCs via flow cytometry.

442

443 4.7.1. Sort *h*MPCs on a flow cytometer with 100 μm nozzle at a pressure of 20 psi. Determine live
444 *h*MPCS based on side and forward scatter as well as 7-AAD negativity (sorting parameters and a
445 representative sort can be seen in **Figure 3**).

446

447 4.7.2. Cells positive for PE-Cy7 (780/60 nm) and AlexFluor488 (530/30 nm) represent
448 CD56$^+$/CD29$^+$ cells and thus are Pax7 positive cells (*h*MPCs). Sort double positive cells into
449 collection tubes containing a 300 μL cushion of FACS buffer.

450

451 NOTE: The purity of sorted cultures can be determined by immunostaining for Pax7 followed by
452 downstream flow cytometry analysis (**Figure 4**).

453

454 4.7.3. Discard all other cells.

455

456 4.7.4. Transfer the sorted cells to a 15 mL conical tube.

457

458 4.7.5. Spin the *h*MPC suspension at 300 x *g* for 5 min at RT.

459

460 4.7.6. Carefully aspirate the supernatant and resuspend in 1 mL of GM.

461

462 4.7.7. Count cells using a hemocytometer or an automated cell counter.

463

464 NOTE: While the number of cells may be inferred as the number of events that are positively
465 sorted, this count has been found to overestimate the number of cells by 10–40% compared to
466 traditional cell counting methods.

467

468 4.7.8. Seed the *h*MPCs as outlined in step 3.2.11.

469

470 NOTE: Verification of the purity of *h*MPC population may be determined by immunostaining for
471 Pax7 and analysis via flow cytometry.

472

473 **5. Characterizing hMPC cultures using an imaging cytometer**

474

475 5.1. Use the imaging cytometer (**Table of Materials**) to perform confluence scans.

476

477 5.1.1. Select **Create New Scan** and then enter/select the **Plate Category**, **Plate Profile** and **Plate**
478 **ID**. The imaging cytometer can accommodate 96, 24, and 6 well plates.

479

480 5.1.2. Under **Application**, select **Confluence > Confluence 1**.

481

482 5.1.3. Select a well to view using the navigator on the right of the screen.

483

5.1.4. Find a plane of focus where the cells appear white compared to the background and select **register manual** or allow the imaging cytometer to select the plane of focus by selecting **register auto**.

NOTE: For the 96 well plates suggested in the **Table of Materials**, an approximate position is 4.3 units.

5.1.5. Use the mouse to highlight all wells that need to be scanned. The entire well area will automatically be scanned. Select **Start Scan**.

5.1.6. Select analysis settings optimal for plate and cell type.

NOTE: For *h*MPCs, the following settings are optimal: intensity = 6, saturated intensity = 0, diameter = 8, minimum thickness = 3, min cluster size = 500, and precision = high.

5.1.7. Select **Start Analysis**.

NOTE: Visually inspect images to ensure that the user and imaging cytometer agree regarding the perimeter of cells. If a large discrepancy exists, rescan the plate using a different plane of focus or different analysis settings. Confluence scanning can be used to compare growth between cultures, time the administration of a treatment, or to monitor cells and decide when to initiate differentiation. If differentiation is desired, details are provided in section 6.

5.2. Use the imaging cytometer to count cells.

5.2.1. Prepare a staining solution containing 12 mL of Ham's F12, 6 μL of Hoechst 33342 and 24 μL of propidium iodide.

5.2.2. Aspirate media and replace with staining solution, incubate at 37 °C for 15 min.

5.2.3. Aspirate staining solution and replace with Ham's F12.

NOTE: It is important to use a media with low levels of phenol red to obtain clear images. Alternatively, PBS may be used.

5.2.4. Load the plate onto the imaging cytometer and under **Application**, select **Cell Viability** > **Live + Dead + Total**. The live channel will be a bright field image, the dead channel will be the propidium Iodide, and the total channel will be the Hoechst 33342.

5.2.5. Set the **Live** channel to bright field and click **Register Auto** under **Focus Setup**.

5.2.6. Under the **Total** channel, set the channel to blue. Use **Find focus** to bring the nuclei into focus or use the up and down arrows to manually set the focus. Click **Set offset** to select the focus.

528
541
542 **6. Differentiating *h*MPCs to myotubes**
543
544 6.1. Determine the hMPC confluence using the protocol in section 5.1. To differentiate hMPCs
545 into myotubes, it is ideal for cells to be 80–85% confluent as determined by the confluence
546 function on the imaging cytometer (see section 5). If hMPCs from more than one unique donor
547 are used, confluence scanning allows cells from different donors to begin differentiation at the
548 time they reach confluence.
549
550 NOTE: At 80% confluence, there are approximately 66,000 cells/cm^2.
551
552 6.2. Wash cells 2x with differentiation media (97% Ham's F12, 2% equine serum, 1%
553 penicillin/streptomycin, pH 7.4, passed through a 0.22 µm filter).
554
555 6.3. Maintain cells in differentiation media for as long as the experiment dictates while switching
556 media daily.
557
558 6.4. Directly assess myotube formation by immunostaining for embryonic myosin heavy chain,
559 which is typically detectable after 7–10 days of differentiation.
560
561 NOTE: The time required to form myotubes may differ slightly depending on donor and number
562 of cells when differentiation was initiated; therefore, it is recommended that cultures from each
563 donor be monitored individually.
564
565 **REPRESENTATIVE RESULTS:**
566 Representative flow cytometry results of *h*MPC isolation from human muscle tissue can be
567 viewed in **Figure 1**. *h*MPCs can be identified by first gating events based on side scatter and
568 forward scatter to eliminate dead cells or debris, followed by selecting only cells which are
569 negative for 7-AAD and therefore are viable. Selection of cells positive for both the cell surface
570 markers CD56 and CD29 represents the *h*MPC population. A biopsy of 60 mg only provides
571 approximately 75–250 *h*MPCs.

572

573 A representative confluence scan is shown in **Figure 2A**. The green outlines in **Figure 2B** were

574 generated by the imaging cytometer and show how the imaging cytometer determined

575 confluence based on the analysis settings selected. In both images shown, the confluence

576 determined by the imaging cytometer agrees with the confluence visually determined by the

577 user. However, these images also highlight that the imaging cytometer is not perfect. For

578 example, the red arrow highlights an imperfection in the plate which is being counted as cells. If

579 a large number of these imperfection are present on the plate, the resulting confluence will not

580 accurately represent the confluence of the cells. **Figure 2C** shows a representation of all three

581 channels used to count cells (brightfield, blue and red) and the merged image. **Figure 2D** shows

582 heterogeneity between donors. Discovering and accurately characterizing this heterogeneity is a

583 key application of the imaging cytometer. **Figure 2E** shows that confluence measurements and

584 nuclei counts from unique donors are highly correlated.

585

586 **Figure 3** provides a guideline for how to identify *h*MPCs based on the FACS procedure described

587 in this protocol. Gating based on forward and side scatter allows for separation of viable cells

588 from debris (**Figure 3A**). A comparison of forward scatter by height to forward scatter by area

589 was used to distinguish events representing single cells (the boxed area in **Figure 3B**). Viable cells

590 are marked by a lack of incorporation of the viability stain 7-AAD (**Figure 3C**). Finally, cells positive

591 for both CD56 and CD29 are identified as *h*MPCs (**Figure 3D**). To validate the FACS procedure

592 described in this protocol the selection of Pax7 positive cells can be determined by

593 immunostaining cells with a Pax7-specific antibody and measuring expression on a flow

594 cytometer. **Figure 4** compares the number of *h*MPCs as determine by Pax7 immunostaining

595 (**Figure 4A**) vs. the FACS procedure (**Figure 4B**) detailed in this protocol from the same population

596 of cells. **Figure 5** uses Pax7 immunostaining and analysis via flow cytometry to show the

597 enrichment of Pax7 expressing cells in the total population after FACS (**Figure 5A** compared to

598 **Figure 5B**) as well as the maintenance of the number of Pax7 expressing cells after passaging

599 (**Figure 5B** compared to **Figure 5C**).

600

601 *h*MPCs can be differentiated to form myotubes by following section 6. To determine whether

602 isolated *h*MPCs maintain myogenic capacity cells can be immunostained with an antibody specific

603 for embryonic myosin heavy chain and visualized using fluorescent microscopy. Representative

604 images of embryonic myosin heavy chain positive myotubes can be viewed in **Figure 6**.

605

606 **FIGURE LEGENDS:**

607

608 **Figure 1: Representative flow cytometry images for hMPCs sorted directly out of muscle biopsy**

609 **tissue.** (**A**) Side scatter area (y-axis) vs. forward scatter area (x-axis) gating strategy used to

610 identify all cells in a donor sample. (**B**) 7-AAD viability stain (y-axis) vs. forward scatter (x-axis) to

611 identify live cells. (**C**) Negative selection markers (y-axis) vs. the negative selection marker CD34.

612 (**D**) Negative selection marker CD34 (y-axis) vs. the positive selection marker CD29. (**E**) Positive

613 selection marker CD56 (y-axis) vs. the positive selection marker CD29 (x-axis). (**F**) Yields from

614 three different skeletal muscle biopsies. FSC, forward scatter; SSC, side scatter; *h*MPCs, human

615 muscle progenitor cells.

616

Figure 2: Proliferative potential of *h*MPCs derived from human donors is maintained after 6 passages. (**A**) Representative confluence scan. (**B**) Representative confluence scan with green outlines showing how the imaging cytometer determined confluence. The red arrow shows an imperfection on the plate. (**C**) Representative brightfield, Hoechst 33342 (blue), and propidium Iodide (red) staining and a merged image of these three channels. Scale bars indicate 1mm. (**D**) Nuclei counts from 6 unique donors highlights *h*MPC heterogeneity. (**E**) Confluence and nuclei count are highly correlated.

Figure 3: Representative flow cytometry sorting parameters for *h*MPC isolation. (**A**) Side scatter area (y-axis) vs. forward scatter area (x-axis) gating strategy used to identify all cells in a donor sample. (**B**) Forward scatter height (y-axis) vs. forward scatter area (x-axis) to disqualify doublets from the sorting population. (**C**) Forward scatter height (y-axis) vs. 7-AAD incorporation to identify viable cells. (**D**) PE-Cy7 (CD56) vs. AF488 (CD29) staining with Q2 representing double positive cells (*h*MPCs). Color represents the density of events.

Figure 4: Comparison of CD29/CD56 positivity vs. Pax7 positivity in passage 4 hMPCs from the same donor. (**A**) Count (y-axis) vs. Pax7 expression (x-axis). (**B**) Positive selection marker CD56 (y-axis) vs. positive selection marker CD29 (x-axis). NCAM, neural cell adhesion molecule; hMPC, human muscle progenitor cell.

Figure 5: Pax7 positivity is maintained in hMPCs derived from the same donor over multiple passages. (**A**) Pax7 expression (x-axis) of cells which had been passaged 4 times prior to FACS sorting. (**B**) Pax7 expression (x-axis) of hMPCs 1 passage after FACS sorting for CD29 and CD56 positivity (5 total passages). (**C**) Pax7 expression (x-axis) of hMPCs 2 passages after FACS sorting for CD29 and CD56 positivity (6 total passages).

Figure 6: Staining of *h*MPC derived myotubes for embryonic myosin heavy chain. Representative microscopic images of differentiated *h*MPCs co-stained with DNA stain (Hoechst 33342, blue) and embryonic myosin heavy chain (green) (n = 3).

DISCUSSION:

Primary *h*MPCs are an important research model used to understand skeletal muscle biology and the regenerative process. Additionally, *h*MPCs have the potential to be used for therapy. However, there are recognized challenges in using primary *h*MPCs for both research and therapy, including the limited understanding of cells derived from humans[10]. There is also a large degree of variation in the expansion capacity among donor cultures, which limits the potential for use of *h*MPCs and can affect research results[11]. For example, it has recently been demonstrated that *h*MPCs isolated using the method described in this protocol produced cells that varied in expansion capacity and this was driven by transcriptional profile that did not align with sex or age[11].

Here, we present an efficient and high yield method for isolating *h*MPCs in sufficient quantities for a number of downstream applications including differentiation into myotubes, thereby

660   modeling the myogenic process in vitro. Our method relies on FACS to isolate $CD56^+$/$CD29^+$ cells,
661   which have previously been identified as representing an enriched Pax7 expressing population[12].
662   Other equally valid cell sorting strategies have also been described[5,6].
663
664   The most important aspect of our *h*MPC isolation and culture method is the careful monitoring
665   of individual cultures. The donor-based heterogeneity includes number of *h*MPCs obtained from
666   each biopsy, their time to initiate proliferation in vitro, and their population expansion rate.
667   Therefore, if differences in myotube formation after a treatment is the question of interest
668   individual, *h*MPC cultures should be switched to treatment based on a confluence level
669   determined *a priori* and assessed objectively using the imaging cytometer.
670
671   Our method to isolate and culture *h*MPCs has been optimized to obtain yields of cells in the
672   millions for in vitro experiments from relatively little starting biopsy tissue (50–100 mg). The
673   major benefit of this approach is that multiple assays and experiments can be performed on
674   *h*MPCs from the same donor, allowing for maintenance of donor phenotype across experiments
675   while introducing little inter-experiment variability. Our method differs from recently published
676   methods that focus on extracting the purest population of Pax7 expressing cells directly out of
677   biopsy tissue where the focus is xenotransplantation[8]. The challenge with these previous
678   methods however, is that they require a starting tissue weight of greater than one gram.
679   Therefore, they are not practical for research involving human skeletal muscle biopsies. One
680   limitation of our method is that the potential for xenotransplantation has not been assessed.
681   However, this type of procedure was not one of the original intended downstream applications.
682   Future directions of this method may include assessing the engraftment capacity of *h*MPCs, after
683   undergoing our isolation procedure, to determine whether there is potential for use of this
684   procedure in MPC transplantation studies.
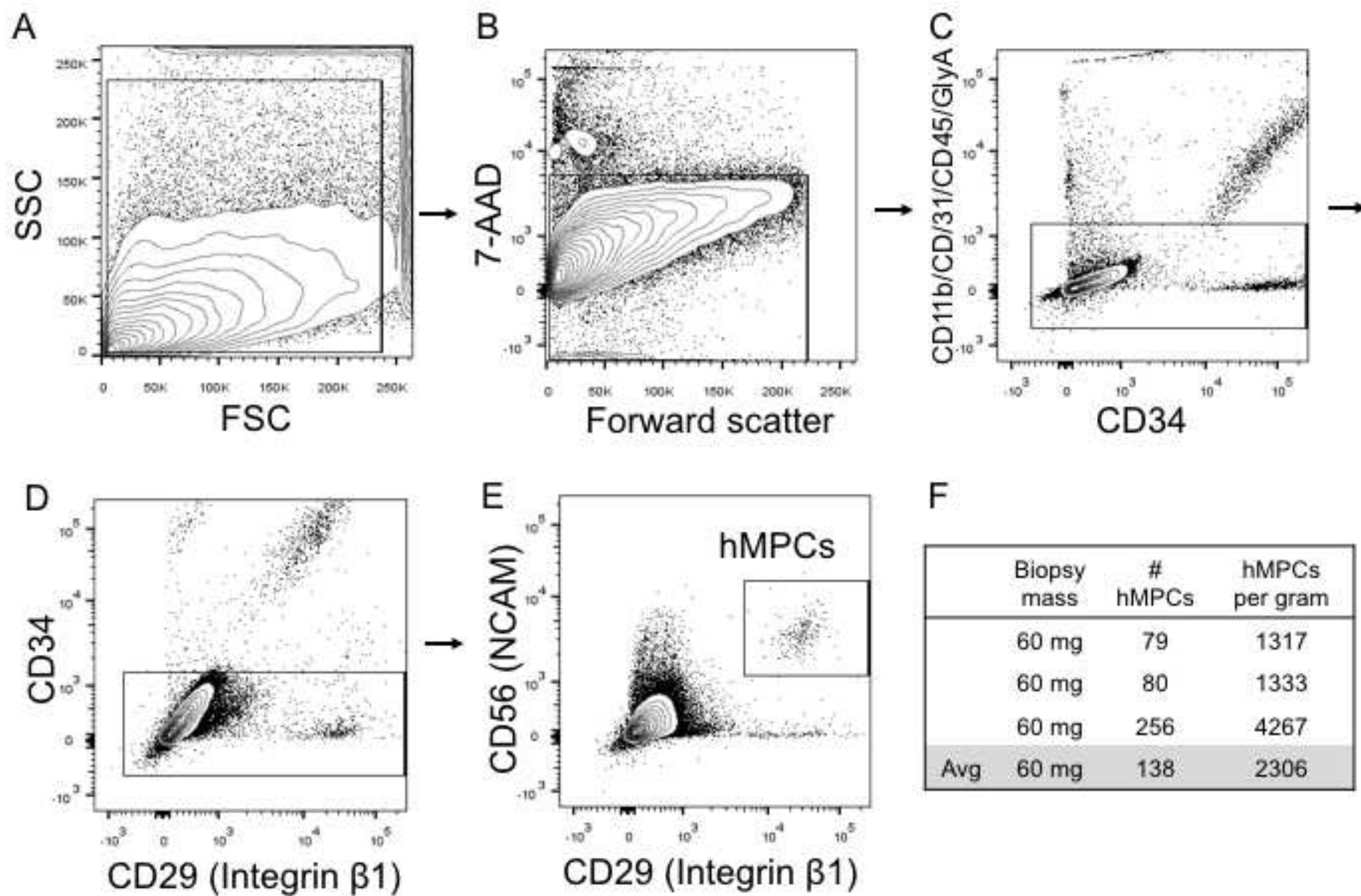685

695

696   **DISCLOSURES:**
697   The authors have nothing to disclose.
698

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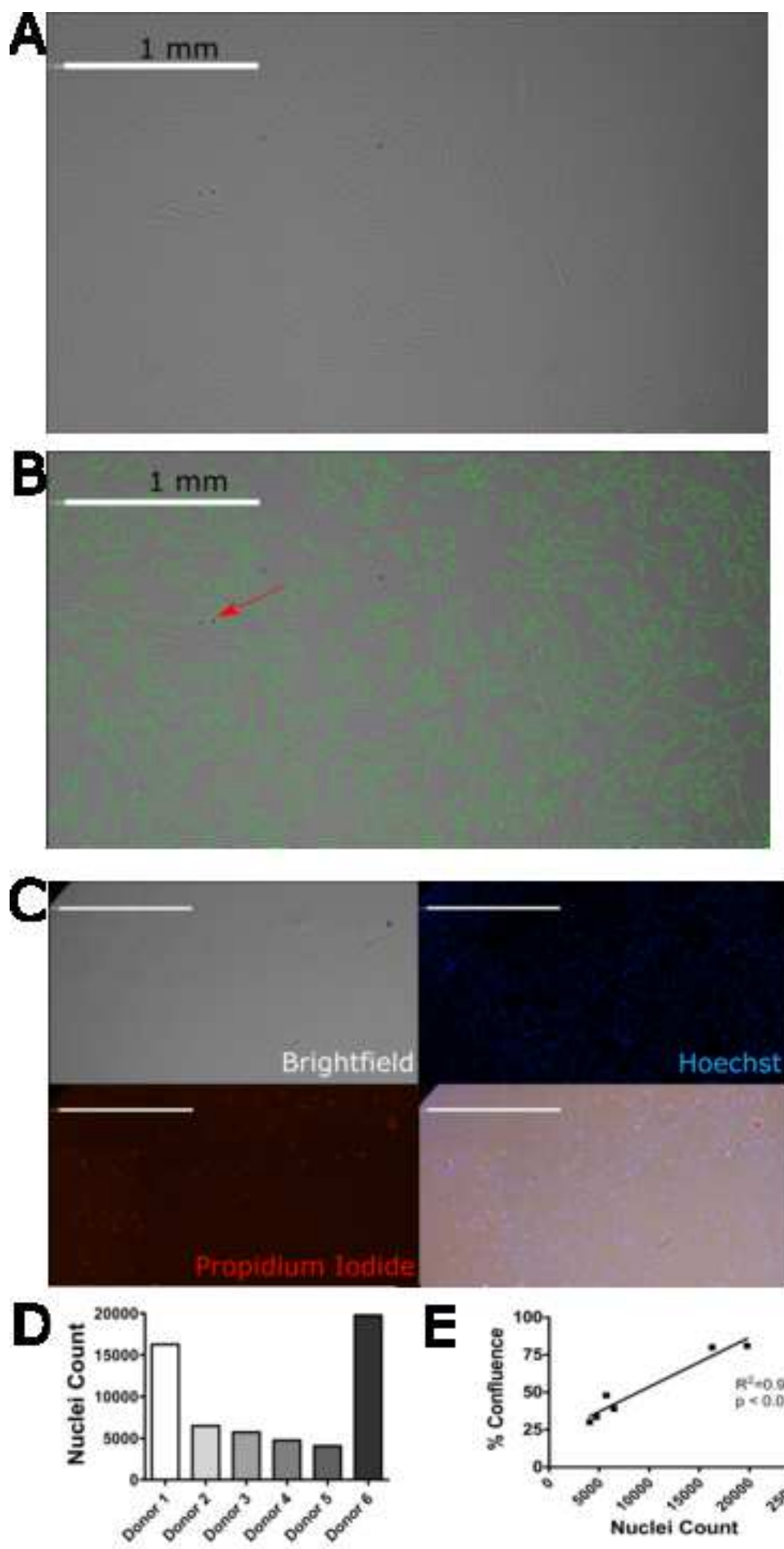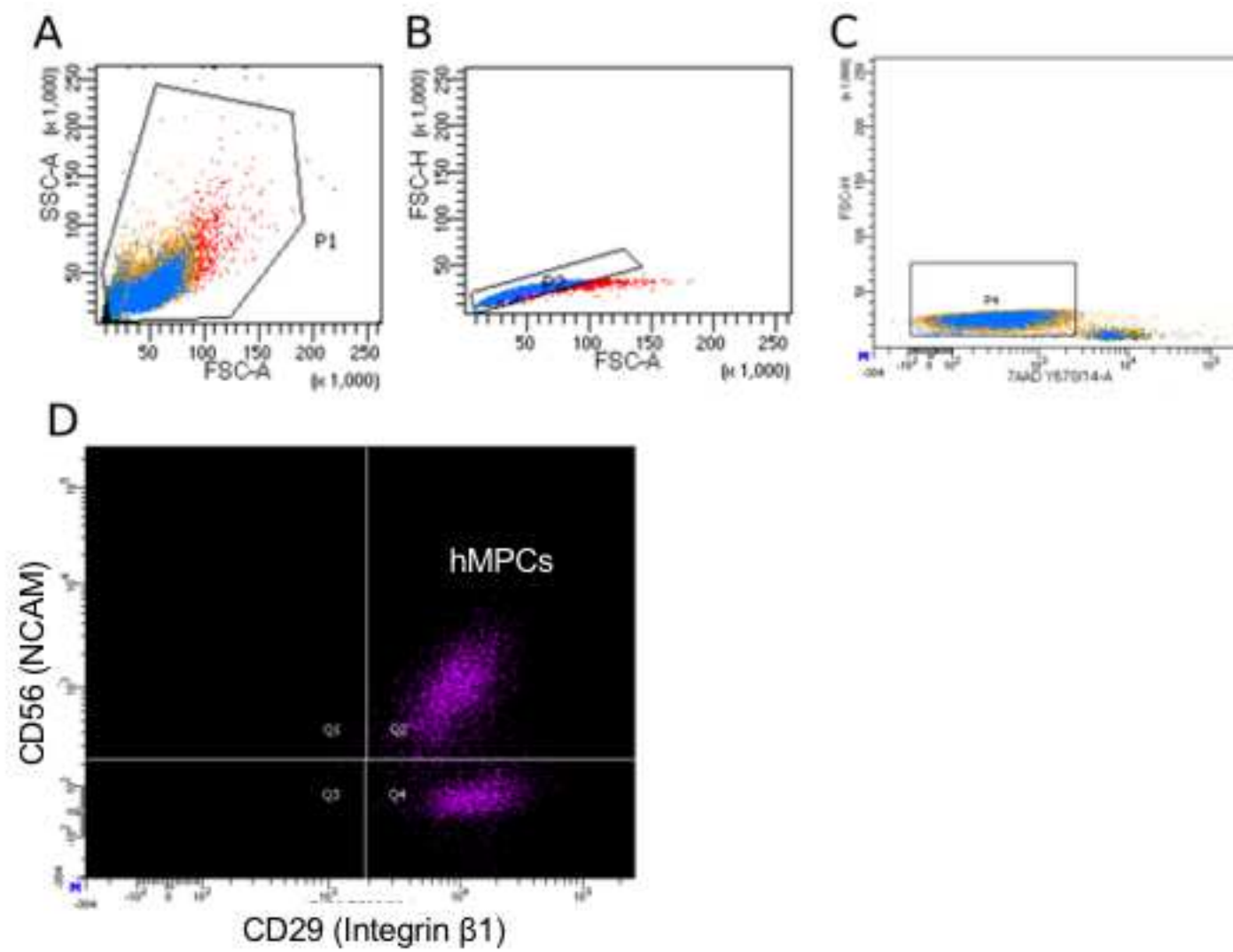726 Muscles. *Stem Cell Reports*. **5** (3), 419–434 (2015).

Figure 1

Figure 1

Figure 2

Click here to access/download;Figure;Figure 2.png

Figure 3

Figure 4
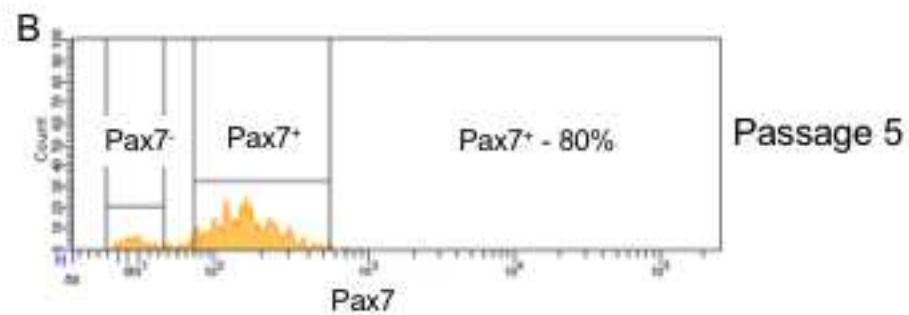
Figure 5

A — Pax7⁻ Pax7⁺ Pax7⁺ - 58% Passage 4

B — Pax7⁻ Pax7⁺ Pax7⁺ - 80% Passage 5

C — Pax7⁻ Pax7⁺ Pax7⁺ - 76% Passage 6

Figure 6
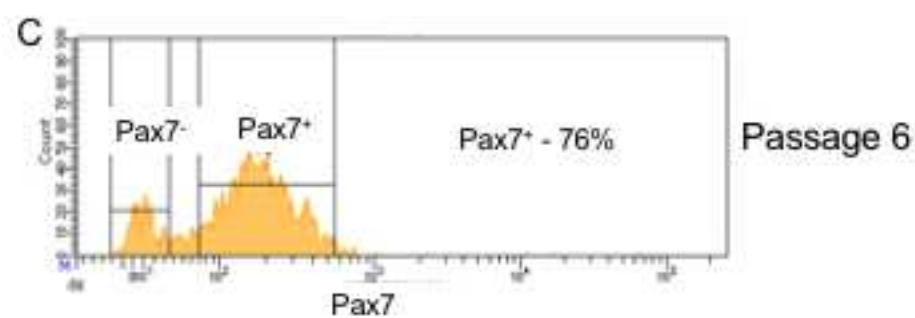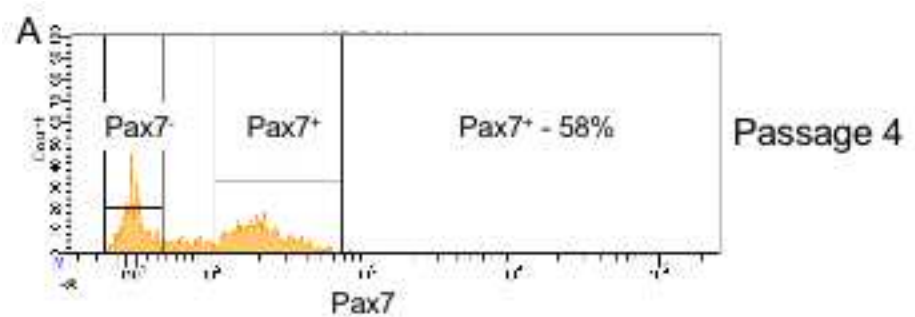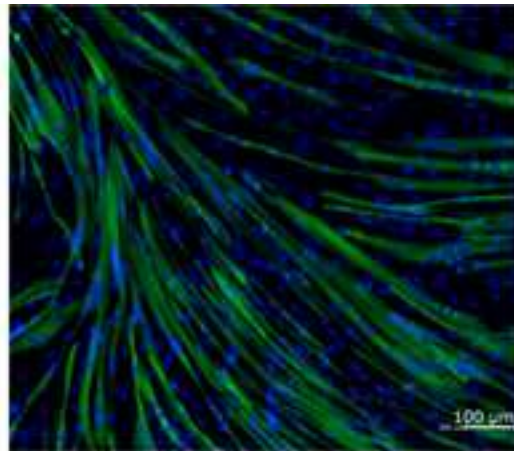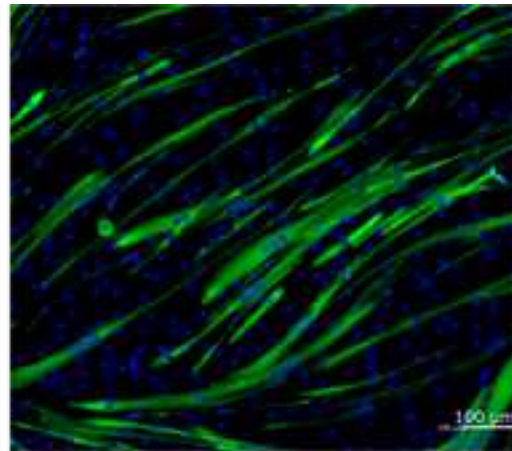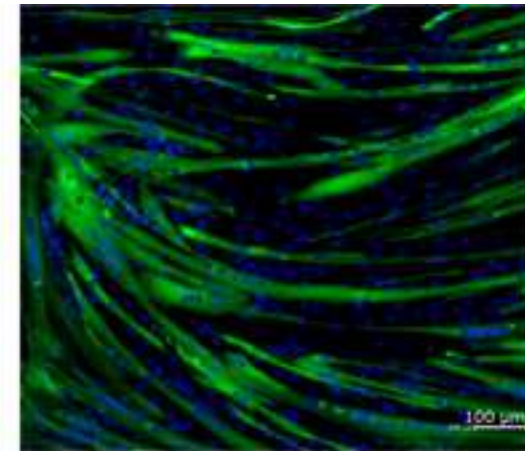
DONOR 1   DONOR 2   DONOR 3

Name of Material/ Equipment	Company	Catalog Number
0.25% Trypsin, 2.21 mM EDTA	Corning	25-053-Cl
10 cm cell culture plate	VWR	664160
15 mL Falcon tube	Falcon	352196
24 well cell culture plate	Grenier Bio-One	662 160
7-AAD Viability Staining Solution	eBioscience	00-6993-50
Alexa Fluor 488 anti-human CD29, Clone: TS2/16	BioLegend	303016
Black 96-well cell culture plate	Grenier Bio-One	655079
Celigo S	Nexcelcom Bioscience	
Cell Strainer	VWR	352350
Collagen Type I (Rat Tail)	Corning	354236
Collagenase D	Roche	11 088 882 001
Dimethyl Sulfoxide	VWR	WN182
Dispase II	Sigma Life Sciences	D4693
Dulbecco's Modified Eagle Medium Low Glucose powder	Gibco	31600-034
Dulbecco's Phosphate Buffered Saline	Gibco	21600-010
EDTA Disodium Salt Dihydrate	J.T. Baker	4040-01
Fetal Bovine Serum	VWR	89510-186
Ham's F12	Gibco	21700-026
Heat Inactivated Equine Serum	Gibco	26-050-070
Hemocytometer	iNCyto	DHC-N0105
Hibernate A	Gibco	A1247501
Hoechst 33342, trihydrochloride, trihydrate	Life Technologies	H21492
Isopropanol	Fisher Scientific	A416P-4
Moxi buffer	Orflo	MXA006
Moxi Cassettes	Orflo	MXC002
Moxi z Mini Automated Cell Counter	Orflo	
Mr. Frosty Freezing Container	Thermo Fisher Scientific	5100-0001
Normal Goat Serum (10%)	Thermo Fisher Scientific	50062Z
PE-Cy7 Mouse Anti-human CD56 , Clone: B159	BD Pharmingen	557747
Penicillin/Streptomycin 100X Solution	Corning	30-002-Cl
Propidium iodide	Thermo Fisher Scientific	P3566

Recombinant Human basic fibroblast growth factor	Promega	G5071
Recovery Cell Culture Freezing Medium	Gibco	12648-010
Sodium Bicarbonate	Fisher Scientific	S233-3
Sterile Round Bottom 5 mL tubes	VWR	60818-565
UltraComp eBeads	eBioscience	01-2222-42

Comments/Description
Trypsin used for removing adherent *h* MPCs from cell culture vessels
Plates used for culturing *h* MPCs
15 mL conical tubes used throughout the *h* MPC isolation and culturing protocols
Plates used for culturing *h* MPCs
Viability stain for identifying living cells during FACS sorting
Conjugated antibody for FACS
96-well cell culture plate ideal for fluorescent imaging using the Celigo S
Imaging cytometer used to track *h* MPC cultures
Cell strainer to eliminate large pieces of debris during muscle biopsy processing
Collagen for coating cell culture plates
Used for degradation of collagen and other connective tissue in the skeletal muscle biopsy tissue
Used for cryopreservation of *h* MPCs
A protease used for enzymatic digestion of skeletal muscle biopsy tissue
Low glucose DMEM for muscle biopsy processing
PBS for muscle biopsy processing
Required for FACS buffer
Fetal bovine serum used for *h* MPC growth media
Base media for *h* MPCs
Horse serum used to make *h* MPC differentiation media
Used to count cells
Media for preserving skeletal muscle biopsy tissue
DNA stain for identifying all cells using the Celigo S
Used for controlled rate freezing of *h* MPCs
Buffer for automated cell counter
Cassesttes for automated cell counter
Automated cell counter
Commerically available controlled rate cell freezing container
Goat serum used in FACS buffer
Conjugated antibody for FACS
Antibiotics added to culture media
DNA stain for identifying dead cells using the Celigo S

Supplement in *h* MPC growth media to prevent spontaneous differentiation
Media used to cryoperseve muscle biopsy slurries
Added to Ham's F12
Tubes used for FACS
Compensation beads fort calibrating flow FACS settings

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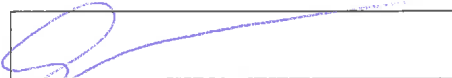Department: Division of Nutritional Sciences

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